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Myosins and DYNLL1/LC8 in the honey bee (*Apis mellifera* L.) brain

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ABSTRACT

Honey bees have brain structures with specialized and developed systems of communication that account for memory, learning capacity and behavioral organization with a set of genes homologous to vertebrate genes. Many microtubule- and actin-based molecular motors are involved in axonal/dendritic transport. Myosin-Va is present in the honey bee *Apis mellifera* nervous system of the larvae and adult castes and subcastes. DYNLL1/LC8 and myosin-IIb, -VI and -IXb have also been detected in the adult brain. SNARE proteins, such as CaMKII, clathrin, syntaxin, SNAP25, munc18, synaptophysin and synaptotagmin, are also expressed in the honey bee brain. Honey bee myosin-Va displayed ATP-dependent solubility and was associated with DYNLL1/LC8 and SNARE proteins in the membrane vesicle-enriched fraction. Myosin-Va expression was also decreased after the intracerebral injection of melittin and NMDA. The immunolocalization of myosin-Va and -IV, DYNLL1/LC8, and synaptophysin in mushroom bodies, and optical and antennal lobes was compared with the brain morphology based on Neo-Timm histochemistry and revealed a distinct and punctate distribution. This result suggested that the pattern of localization is associated with neuron function. Therefore, our data indicated that the roles of myosins, DYNLL1/LC8, and SNARE proteins in the nervous and visual systems of honey bees should be further studied under different developmental, caste and behavioral conditions.

1. Introduction

The honey bee *Apis mellifera* L. is a model organism with a wide behavioral repertoire that serves as a baseline for studies of the complexity of cognitive functions in insect brains (Giurfa, 2003; Menzel, 2001). In addition to its behavioral organization, this honey bee has a set of putative genes that are highly related to vertebrate genes, including most of the genes that encode factors related to cell signaling/signal transduction (Consortium, 2006; Nunes et al., 2004; Sen Sarma et al., 2007). Studies of the honey bee brain have identified genes and proteins that are expressed in this tissue (Calabria et al., 2008; Garcia et al., 2009; Peixoto et al., 2009; Robinson, 2002; Whitfield et al., 2002) and indicated aspects of insect navigation, social behavior and learning processes (Garcia et al., 2009; Kamikouchi et al., 2000; Menzel and Muller, 1996; Robinson et al., 1997; Sen Sarma et al., 2009).

Actin (myosins) and microtubule (dynein and kinesin) -based motors use energy derived from ATP to generate the force required for axonal/dendritic transport of vesicle cargo and growth cone dynamics in neurons (Endow and Titus, 1992; Goodson et al., 1997; Hackney, 1996; Reck-Peterson et al., 2000; Suter et al., 2000; Titu and Gilbert, 1999; Vale, 2003). Myosins (classes II, V and VI), kinesins and dyneins are expressed in vertebrate neural tissues and have been extensively characterized (Hirokawa et al., 2010). Biochemical and immunolocalization data from the honey bee have indicated that motor proteins are present in the brain (Calabria et al., 2010) and synaptosomes (Silva et al.,

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2002), and in photoreceptor cells (Baumann, 1998, 2001). Espindola et al. (2000) identified and partially sequenced the 10-kDa tail domain-associated light chain of myosin-Va (now termed DYNLL1/LC8). This molecule has high homology to the light chain of a 8-kDa dynein isolated from the unicellular alga *Chlamydomonas* sp. as well as a diverse set of proteins, which include cytoplasmic dynein, protein inhibitor of neuronal nitric oxide synthase (PIN) and apoptotic factors (Jaffrey and Snyder, 1996; King, 2008; King and Patel-King, 1995a,b). Indeed, vertebrate brains are an important source of the purification and biochemical characterization of myosin-Va (Cheney et al., 1993; Coelho and Larson, 1993; Costa et al., 1999; Espindola et al., 2000; Nascimento et al., 1996).

The honey bee nervous system is composed of the ocular system, compound eyes, protocerebrum, antennal lobes and mushroom bodies (Nassel et al., 1986). These neuropils require first- and second-order sensory attributes with distinct properties. The intracellular transport of organelles and the exocytosis and endocytosis of large density core vesicles and synaptic vesicles in cells have been shown to involve molecular motors (Langford, 2002; Mermall et al., 1998; Rudolf et al., 2010; Schnapp and Reese, 1989; Schnapp et al., 1992; Yamazaki et al., 1995). Membrane fusion in eukaryotic cells involves several families of evolutionarily conserved proteins, including SNARE and motor proteins (Hirokawa et al., 2010; Ungar and Hughson, 2003).

One of the aims of our study was to identify the orthologs of some of these molecules in the honey bee brain. Monoclonal antibodies for syntaxin, munc18, synaptophysin, CaMKII, clathrin, SNAP25, cytoplasmic dynein intermediate chain and PIN were employed. We also used polyclonal antibodies for myosins -IIb, -Va, -VI and –IXb, and DYNLL1/LC8. Immunoblotting and immunohistochemistry have been used to identify these proteins in the soluble and membrane-enriched vesicle fractions of the honey bee brain as well as in paraformaldehyde-fixed brain sections. Previous studies have also indicated that myosin-Va is found in synaptic vesicle preparations and forms stable complexes between synaptic vesicle membrane proteins (Mani et al., 1994; Prekeris and Terrian, 1997). In the vertebrate brain, 5–15% of the total zinc is concentrated in synaptic vesicles (Frederickson, 1989; Frederickson and Moncrieff, 1994), which has been studied using the Neo-Timm method (Babb et al., 1991). Moreover, zinc serves as an endogenous neuromodulator of several important receptors, including *N*-methyl-D-aspartate (NMDA) (Smart et al., 1994).

Functional studies of honey bee myosin-Va have not been carried out until now. In this study, we addressed the effects of intracerebral injections of melittin and NMDA on the honey bee. Melittin is a polypeptide present in bee venom (Habermann, 1972) and a potent calmodulin antagonist (Steiner et al., 1986). Calmodulin is the most extensively studied member of the intracellular calcium-binding proteins, which includes myosin-Va. Additionally, NMDA is a glutamate-gated ion channel agonist present in both mammals and insects (Paoletti and Neyton, 2007). The NMDA receptor is involved in delayed neuronal death (Choi, 1988) and excitatory synaptic transmission in the central nervous system, which results in learning and memory (Albensi, 2007). A critical role of the NMDA receptor was recently demonstrated in olfactory learning and memory in *Drosophila melanogaster* (Xia et al., 2005) and *A. mellifera* (Locatelli et al., 2005; Si et al., 2004).

The aims of this study were to elucidate some of the biochemical properties and the distribution of myosin-Va and to describe the expression patterns of molecular motors and SNARE proteins in the honey bee (*A. mellifera* L.) brain. Moreover, we evaluated the alterations in myosin-Va expression after intracerebral injections of melittin and NMDA.

2. Materials and methods

2.1. Antibodies

Rabbit affinity-purified polyclonal antibodies were used in this study. Anti-chicken brain myosin-Va (α-myosin-Va) head domain recombinant protein (Espreafico et al., 1992; Suter et al., 2000), anti-pig myosin-VI (α-myosin-VI) tail fusion protein (Hasson and Mooseker, 1994) and anti-myosin-IXb heavy chain tail domain recombinant protein (Post et al., 1998) were all from the Mooseker Laboratory (Yale University, New Haven, CT, USA). Anti-rabbit myosin-IIb (α-myosin-IIb) was produced in the Larsons Laboratory (USP, Ribeirão Preto, SP, Brazil). The dynein light chain (α-DYNLL1/LC8) antibody was generated against the *Chlamydomonas* LC8 recombinant protein (King et al., 1996).

Mouse monoclonal antibodies used included anti-cytoplasmic dynein intermediate chain IC74 (α-DIC; Chemicon International Inc., Temecula, CA, USA); anti-protein inhibitor of neuronal nitric-oxide synthase (α-PIN), anti-calcium/calmodulin-dependent protein kinase II (α-CaMKII), synaptosomal-associated protein 25 (α-SNAP25), anti-protein unc-18 (α-munc18) and anti-clathrin heavy chain (α-clathrin) (Transduction Laboratories, Lexington, KY, USA); α-synaptophysin (Calbiochem, Darmstadt, Germany); and α-synaptotagmin (Sigma Chemical, St. Louis, MO, USA). Secondary antibodies (α-mouse IgG and α-rabbit IgG) conjugated to peroxidase were obtained commercially from Boehringer Mannheim (Mannheim, Germany).

2.2. Biological specimens

Adult honey bees (workers, drones, and queens) were collected from an *A. mellifera* colony (Africanized hybrids) at the experimental garden of the Federal University of Uberlandia (Uberlândia, MG, Brazil). To distinguish between nurse and forager worker honey bees, physical features, i.e., coat condition and damage to wings were considered, as well as the development of the hypopharyngeal gland observed at the time of brain dissections. Pre-pupal honey bee larvaes were collected from *A. mellifera* colonies (Africanized hybrids) and maintained at the experimental apiary of the University of São Paulo (Ribeirão Preto, SP, Brazil). Rabbits and rats used in the assay described in Fig. 1 were provided by the University's Animal Facility and were used under the supervision of the Animal Experiments Review Board at our University.

2.3. Protein extraction

Honey bees were anesthetized on ice and dissected. Larval ganglia and adult brains were removed, frozen in liquid nitrogen, and stored in microtubes at −80 °C. The tissue samples (1 worker/queen or ∼30 worker/drone bee brains, or 2 rabbit/rat brains) were homogenized with a hand blender in cold homogenization buffer (40 mM Hepes, pH 7.7, 10 mM EDTA, 2 mM EGTA, 5 mM ATP, 2 mM DTT, 1 mM benzamidine, 0.1 mM aprotinin and 0.5 mM PMSF). Supernatants were obtained by centrifugation at 40,000*g* for 40 min at 4 °C. When necessary, protein extracts were concentrated by precipitation with 10% trichloroacetic acid for 15 min on ice, which was followed by centrifugation at 12,000*g* for 10 min at 4 °C. The precipitates were then solubilized in a small volume of SDS–PAGE sample buffer (100 mM Tris–HCl, pH 8.0, and 25% glycerol). The optical and antennal lobes, mushroom bodies and central region from thirty honey bee brains were dissected, homogenized and centrifuged as described above. Total protein concentrations (Bradford, 1976) were determined to allow comparison SDS–PAGE and Western blot analyses, as described below.

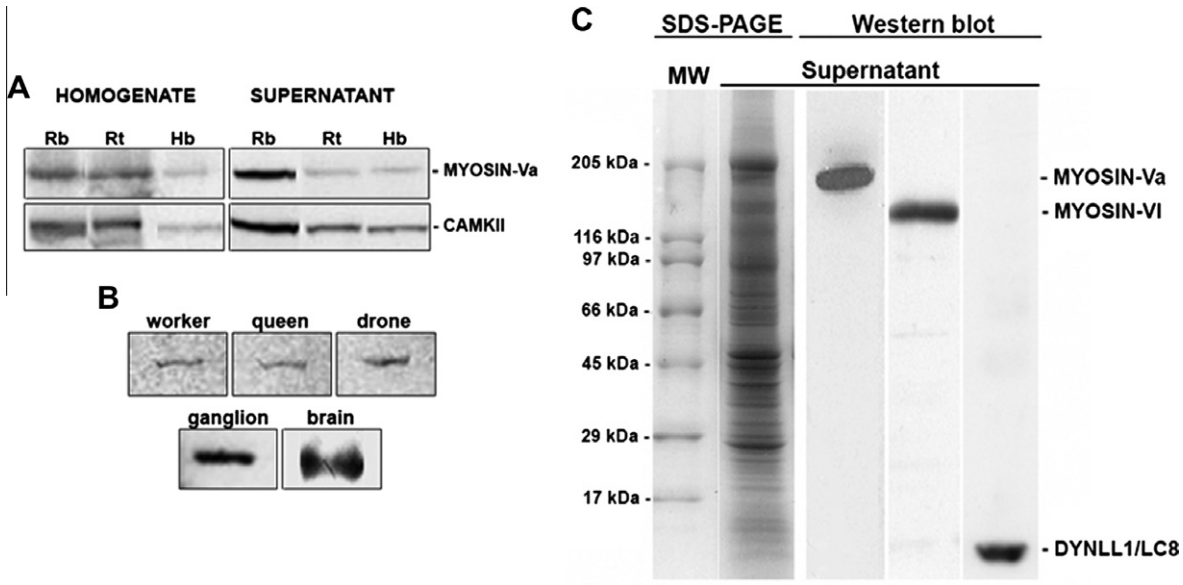

Fig. 1. Immunodetection of myosin-Va in the honey bee *Apis mellifera*. (A) A polyclonal antibody against chicken myosin-Va shows cross-reactivity to the myosin heavy chain in the brain homogenates and supernatants of rabbit (Rb), rat (Rt) and honey bee (Hb) ($n = 1$). A monoclonal antibody against CaMKII alpha subunit from bovine brain also recognizes this subunit in mammals and honey bee brains. (B) Myosin-Va immunodetection in the supernatant samples from adult brain, in the larval ganglion and in different castes (worker, queen and drone) ($n = 1$). (C) Supernatant samples from worker honey bee brains were analyzed by SDS–PAGE gels stained with Coomassie blue and Western blots probed with antibodies against chicken myosin-Va, mouse myosin-VI heavy chain and *Chlamydomonas* dynein light chain (DYNLL1/LC8) ($n = 30$). MW, molecular weight standard.

2.4. SDS–PAGE and Western blot

Total protein samples (20 μg) were applied to 5–22% polyacrylamide gradient gels under denaturing conditions (Laemmli and Favre, 1973). The molecular weight markers were purchased from Sigma–Aldrich (St. Louis, MO, USA), and the gels were stained with Coomassie brilliant blue. For immunoblotting, proteins were transferred to nitrocellulose membranes in Tris–glycine buffer as described by (Towbin et al., 1979). The blots were incubated with 5% dried milk in Tris-buffered saline (TBS-T) (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) and probed with primary antibodies diluted to 0.2 μg/mL in TBS-T and a peroxidase-conjugated anti-rabbit IgG secondary antibody. Antibody binding was detected using an ECL kit (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions, and Kodak film was used for exposure. A chromogenic method using 3,3′-diaminobenzidine tetrahydrochloride (Sigma–Aldrich, St. Louis, MO, USA) as a substrate was also employed. The intensities of the protein bands were analyzed and compared using the Scion Image software, version Alpha 4.03.2 (Scion Corporation, Frederick, MD, USA), and the results were expressed as a percentage of the total content.

2.5. Gel filtration chromatography

In this assay, 40 brains were utilized to obtain the protein fraction that was separated using a Sephacryl S-400 gel filtration column (15 mL volume; Amersham Pharmacia Biotech, Uppsala, Sweden). The column was equilibrated with homogenization buffer and loaded with 3 mg of total protein in a volume of 400 μL. Elution fractions (150 μL) were analyzed by SDS–PAGE and Western blot.

2.6. Solubility assay

Myosin-Va solubility was assessed from protein extracts (Section 2.4), obtained homogenizing honey bee brains with or without 5 mM ATP, and centrifuging homogenates at 40,000*g* for 40 min at 4 °C. The supernatant fractions were analyzed by protein quantification, SDS–PAGE and Western blot.

2.7. Preparation of the myosin-Va-enriched fraction

The myosin-Va-enriched fraction was prepared using the initial fractionation steps of an established protocol for myosin-Va purification (Nascimento et al., 1996). Honey bee brains were homogenized in homogenization buffer at 4 °C, and centrifuged at 40,000*g* at 4 °C for 40 min. The salt concentration of this supernatant (S_1) was increased to 0.6 M NaCl, and the solution was then incubated on ice for 1 h. The pellet (P_2) and supernatant (S_2) were separated by centrifugation of the salt-treated S_1 at 40,000*g* at 4 °C for 40 min. The fractions obtained were analyzed by total protein content, SDS–PAGE and Western blot.

2.8. Cresyl violet and Neo-Timm staining

Brains were fixed in Carnoy solution (ethyl alcohol:chloroform:glacial acetic acid, 60:30:10 by volume) with 1.2% (w/vol) sodium sulfate for 90 min, dehydrated and paraffin-embedded. Eight-micrometer sections were incubated in cresyl violet solution (0.5% (w/vol) cresyl violet, 1 M sodium acetate, and 1 M acetic acid, pH 3.9) for 30 min or incubated in a solution containing 120 mM citrate buffer, 36% (w/vol) arabic gum, 100 mM hydroquinone and 0.08% (w/vol) silver nitrate for 30 min at 35 °C (Babb et al., 1991). The sections were then dehydrated and mounted with Permount (Fisher Scientific, Fair Lawn, NJ, USA).

2.9. Immunohistochemistry

Brains were dissected, fixed in 4% paraformaldehyde, and paraffin-embedded (McLean and Nakane, 1974). Five-micrometer sections were cut and mounted on gelatin-chromium potassium sulfate (chromealum)-coated microscope slides. After antigen retrieval using 10 mM citrate buffer (pH 6.0), antibody detection in

the tissue sections was performed according to Calabria et al. (2010) and Martins et al. (1999). Then, the sections were incubated with H_2O_2 in phosphate-buffered saline (PBS), pH 7.4, for 15 min, followed by a 4 h incubation in 0.02 M sodium phosphate buffer, pH 7.4, containing 450 mM NaCl, 0.2% (w/vol) Triton X-100 and 15% (vol/vol) normal goat serum (blocking buffer). The sections were incubated overnight with antibodies (5 μg/mL) in blocking buffer and washed with PBS containing 0.2% (w/vol) Triton X-100, after each incubation. Endogenous biotin was blocked using a biotin blocking system (Dako Corporation, Glostrup, Denmark). The sections were then incubated for 30 min with biotinylated secondary antibody, diluted 1:200 (vol/vol) in blocking buffer. Biotinylated secondary antibodies were detected using the Elite ABC kit with diaminobenzidine (Vector Laboratories, Burlingame, CA, USA) as the chromogen. All incubations were carried out at room temperature. Sections were mounted with Permount and analyzed using a Reichert Polyvar binocular photomicroscope (Leica, Wien, Austria). Negative controls consisted of sections that were not stained with the primary antibodies. Other sections were stained with hematoxylin and eosin (H&E staining), and mounted in Canada balsam.

2.10. Injections

NMDA (0.04 nmol/μL) and melittin (100 mg/mL) were dissolved in saline and 20 mM Hepes buffer, pH 7.4, containing 1 M NaCl, 1 mM EGTA and 1.2 mM $CaCl_2$, respectively. These reagents were then desalted using Sephadex G-10 resin (Pharmacia Biotech, Uppsala, Sweden), equilibrated with buffer, as described above, and stored. This stock was dissolved fourfold in saline. Groups of worker honey bees were caught before the experiments, maintained in small box at room temperature, and treated with each drug. The head injection site was the clypeus, and each honey bee received 0.1 μL of NMDA or melittin. A control group received saline. A response was counted only if the proboscis was fully extended and extension occurred shortly after stimulus onset. Only honey bees showing this behavioral response were included in the data analysis and brains were dissected after 1, 2, and 3 h. Brain homogenates were prepared individually, and immunoblotted for myosin-Va. All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.11. Statistical analyses

The data of densitometry relating to myosin-V expression in honey bee brain after injection were initially analyzed by one-way ANOVA. When ANOVA analyses detected differences, sets of control and treated groups of animals were compared using *t*-test to determine if the differences were statistically significant. The level of significance was set at $p < 0.05$ in all cases.

3. Results

3.1. Immunodetection of myosin-Va in the honey bee brain

Western blot analyses of rabbit, rat and bee brain homogenates and supernatants with myosin-Va and CaMKII antibodies resulted in the detection of 190 and 60 kDa polypeptides, respectively, in all samples (Fig. 1A). Equal levels of cross-reactivity were observed for the immunodetection of myosin-Va in larval ganglia and brain homogenates of adult worker bees, queens and drones (Fig. 1B). By Western blot, we also observed cross-reaction between myosin-Va (190 kDa), myosin-VI (140 kDa) and DYNLL1/LC8 (10 kDa) in the supernatant fraction of honey bee brains (Fig. 1C).

3.2. Partial fractionation of honey bee myosins and DYNLL1/LC8

To separate the honey bee brain proteins that were immunoreactive to myosins -Va and –VI, and DYNLL1/LC8 by gel filtration chromatography, the supernatant fraction was loaded onto a Sephacryl S-400 column. Immunodetection of the eluted fractions after chromatographic separation showed partial fractionation of myosins -Va and -VI in the early eluted fractions (Fig. 2) whereas DYNLL1/LC8 immunodetection revealed that it was present in most of the elutions.

3.3. Effect of ATP on the solubility of honey bee myosin-Va and DYNLL1/LC8

To investigate the effects of ATP on the solubility of the myosin-Va and DYNLL1/LC8 immunoreactive proteins in the supernatant fraction of the honey bee brain, SDS–PAGE and Western blot were employed (Fig. 3). The SDS–PAGE protein profiles of the supernatant and pellet fractions in the presence and absence of ATP were strikingly similar, and most of the proteins remained in the pellet fraction. However, Western blot revealed that the distribution of myosin-Va in these fractions was different under the two conditions. In the absence of ATP, most of the myosin-Va remained in the pellet, whereas in the presence of ATP, it was partially solubilized. Moreover, the anti-DYNLL1/LC8 blot revealed that this protein was distributed between the supernatant and pellet fractions in the absence of ATP and that the protein level in the soluble fraction was also increased when ATP was present.

3.4. Actin- and microtubule-based molecular motors, SNARE proteins and PIN

Immunoblotting analyses of the honey bee brain supernatant fraction with antibodies against SNARE proteins (SNAP25, munc18, synaptophysin and clathrin), DIC, PIN, and myosins -IIb and -IXb showed the recognition of polypeptides that migrated in SDS–PAGE with relative molecular masses that correspond for each of these proteins (Fig. 4).

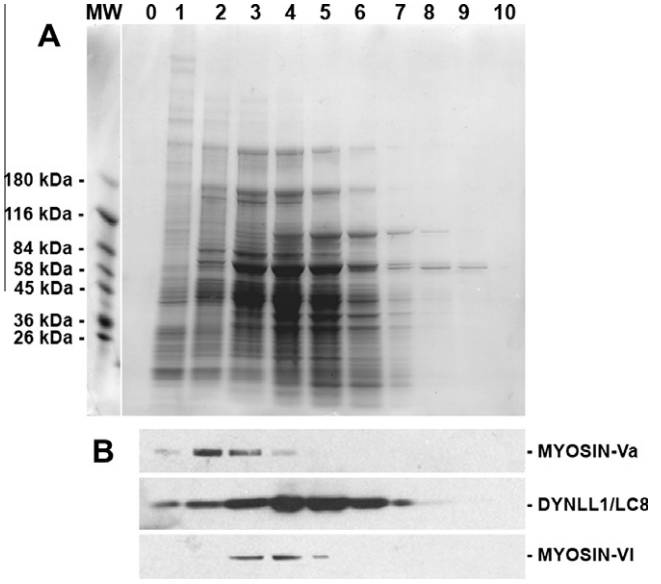

Fig. 2. Partial fractionation of honey bee myosins and DYNLL1/LC8 by gel filtration chromatography (Sephacryl S-400). (A) Protein profile of the eluted fractions of the brain supernatant. (B) Fractions were probed with antibodies to myosin-Va and -VI, and DYNLL1/LC8. MW, molecular weight standard.

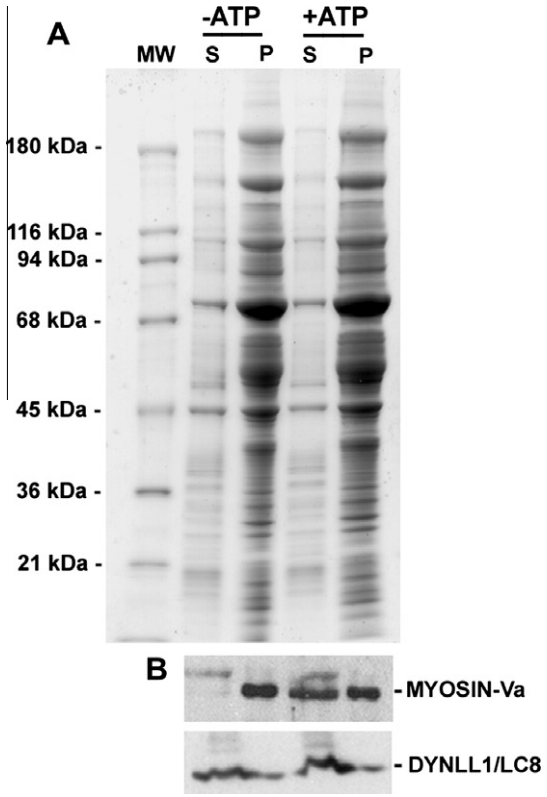

Fig. 3. The effect of ATP on the solubility of honey bee myosin-Va and DYNLL1/LC8. The influence of ATP on myosin-Va solubility was estimated after incubating the protein in the absence (−ATP) and presence of 5 mM ATP (+ATP). The addition of 5 mM ATP to the extracts induced the solubilization of most of the myosin-Va, but not for DYNLL1/LC8. Coomassie blue-stained gel (A) and Western blot (B) loaded with the supernatant (S) and pellet (P) fractions obtained by 40,000*g* centrifugation. MW, molecular weight standard.

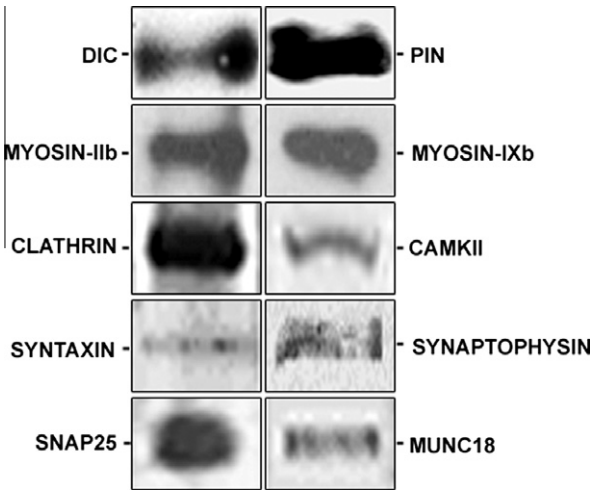

Fig. 4. Actin- and microtubule-based molecular motors [dynein intermediary chain (DIC), myosin-IIb, -IXb heavy chain], SNARE proteins (clathrin, CaMKII, syntaxin, synaptophysin, SNAP25, munc18), and protein inhibitor of nitric oxide synthase (PIN), which is an equivalent of DYNLL1/LC8, were immunodetected in the supernatant of the worker honey bee brains ($n = 30$).

3.5. Myosin-Va is enriched in the pellet fraction of the honey bee brains

Vertebrate myosin-Va is enriched in the pellet fraction of the brain (Evans et al., 1998). Therefore, myosin-Va expression in the P_2 fraction, which is enriched with membranes, actin filaments, organelles and synaptic vesicles, of the honey bee brain was investigated using the strategy illustrated in Fig. 5A. Although the electrophoretic pattern of the Western blot did not reveal an enrichment of proteins in the P_2 fraction, a high ionic strength precipitate of myosin-Va was present in the honey bee brain (Fig. 5B). The Western blot showed strong labeling of myosin-Va in this fraction compared to the S_2 fraction. Furthermore, we observed an enrichment of the anti-DYNLL1/LC8 immunoreactive protein in the P_2 fraction. SNARE proteins, such as clathrin, CaMKII and synaptotagmin, were also observed in the P_2 fraction (Fig. 5B).

3.6. Myosin-Va expression after injection of melittin and NMDA

The potential differences in the expression levels of myosin-Va in nurse and forager worker honey bee brains were observed after injections of the calmodulin antagonist melittin and the glutamate receptor agonist NMDA. Western blot of the supernatant samples from honey bee brain homogenates showed immunoreactivity towards the anti-myosin-Va heavy chain (Fig. 6A), which was quantified by densitometry (Fig. 6B) and revealed substantially higher levels of myosin-Va in nurse brains 2–3 h after administration of melittin or NMDA compared to control (57% and 61% increases, respectively). Melittin treatment induced similar increase in forager worker brains (56%).

3.7. Expression of myosins, DYNLL1/LC8 and CaMKII in brain regions

The main honey bee brain regions, including the mushroom bodies, the central region, and the antennal and optical lobes (Fig. 7B), were dissected and homogenized for analyses of the protein profiles by SDS–PAGE and immunodetection of myosins, DYNLL1/LC8 and CaMKII (Fig. 7A). The homogenates of each dissected honey bee brain region showed similar patterns on SDS–PAGE for most polypeptides; however, some bands were distinctly observed in certain regions. Western blot analysis revealed that myosins -Va and -VI were equally distributed in all regions but showed lower intensity in the mushroom bodies. For DYNLL1/LC8, there was a similar pattern of expression in all regions, but the intensity of CaMKII was lower in the central region (Fig. 7A).

3.8. Distribution of myosins, DYNLL1/LC8 and synaptophysin in the brain regions

To examine the immunohistological localizations of myosins -Va and -VI, DYNLL1/LC8 and synaptophysin in specific honey bee brain regions, we compared tissue sections from the optical lobe, antennal lobe and mushroom bodies by staining with H&E, cresyl violet, and Neo-Timm histochemistry.

We investigated the distribution of myosin-Va and DYNLL1/LC8 in the optical lobe. H&E staining (Fig. 8A and C) showed the optical lobe and its structures, such as the retina, lamina, fenestrated layer, outer chiasm, medulla and lobula. Antibodies that were immunoreactive to myosin-Va (Fig. 8B) and DYNLL1/LC8 (Fig. 8D) recognized these proteins in the monopolar neurons of the fenestrated layer and the cells of the outer chiasm. DYNLL1/LC8 also showed intense staining of the inner chiasm. Myosin-VI was also immunolocalized to the optical lobe (Fig. 9C), where synaptophysin, another known member of the vesicle trafficking apparatus of neurons, (Fig. 9D) was immunolocalized particularly in the retina and lamina. In the optical lobe, we identified both proteins that labeled both the monopolar neurons orderly located in the cell bodies of the lamina and those along the axons in the fenestrated layer. Moreover, we observed weak immunoreactivity of anti-synaptophysin in the fibers of the medulla and outer chiasm. Neo-Timm histochemistry allowed the visualization of the long

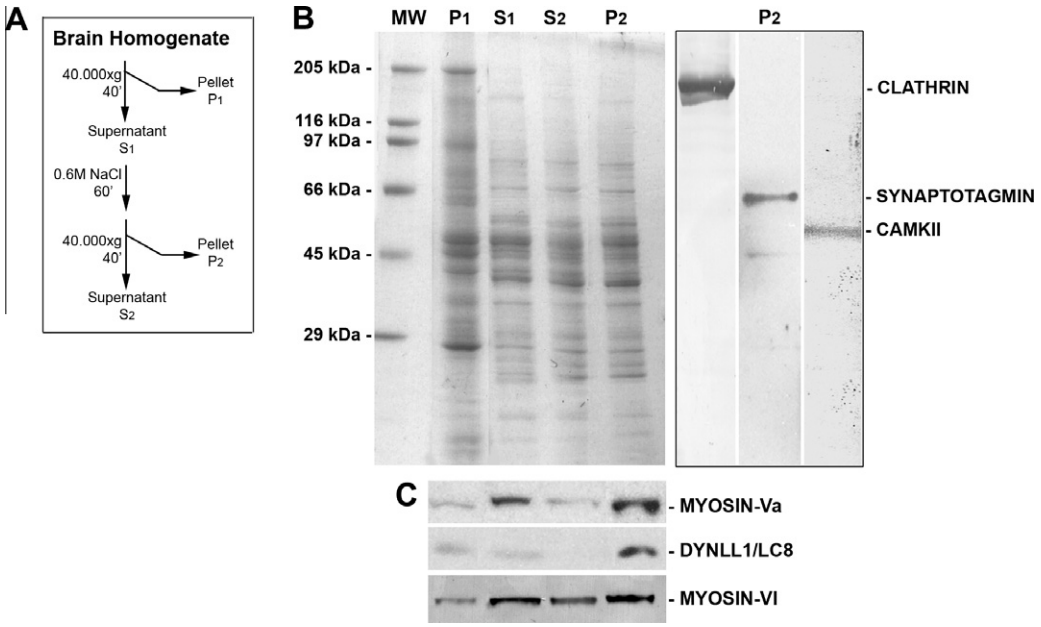

Fig. 5. Honey bee myosin-Va is enriched in the P_2 fraction. (A) Outline of the early steps of the myosin-Va purification protocol (see Section 2; Nascimento et al., 1996). A Coomassie blue-stained gel (B) and Western blot (C) loaded with equal amounts of protein from the fractions shown in the flow chart in (A). The Western blot shows the immunodetection of myosins and DYNLL1/LC8 in these fractions. In the right panel, the P_2 fraction also cross-reacted with antibodies against clathrin, synaptotagmin and CaMKII. MW, molecular weight standard.

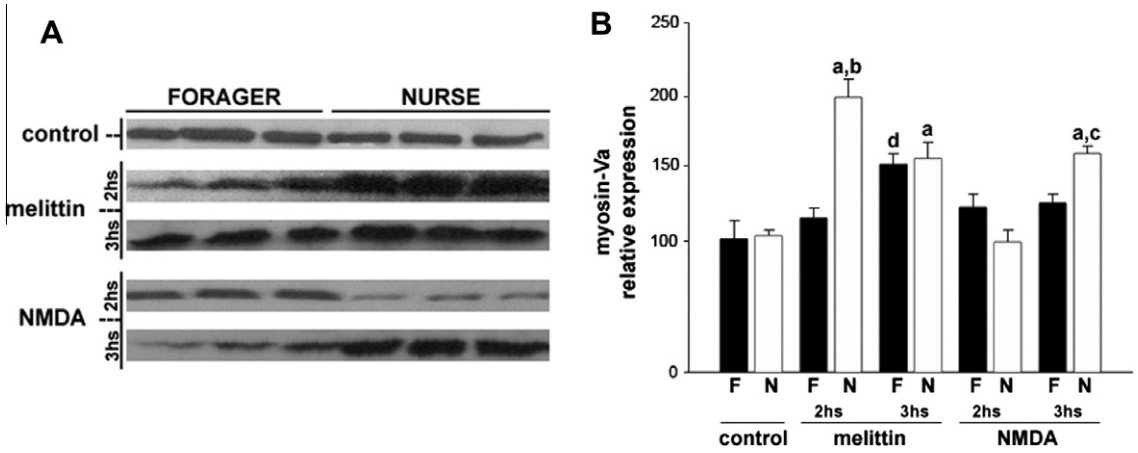

Fig. 6. Myosin-Va expression in forager and nurse honey bee brains after injection of melittin and NMDA. Western blots (A) and quantification (B) of myosin-Va expression in brain extracts from forager (F) and nurse (N) honey bees. Histograms show myosin-Va expression levels on -melittin and -NMDA treated brains relative to vehicle-treated controls. Percentage values were obtained from densitometry analysis of Western blot bands in A. Reference controls were attributed a value of 100. The time (2 and 3 h) and drug (melittin and NMDA) conditions were compared. Data are expressed as means ± SEM, $n = 3$ honey bees/group. (a) N-control vs. N-melittin (2 h), N-control vs. N-melittin (3 h), and N-control vs. N-NMDA (3 h), $p < 0.05$; (b) F-melittin vs. N-melittin (2 h), $p < 0.005$; (c) N-NMDA (2 h) vs. N-NMDA (3 h), $p < 0.005$; (d) F-control vs. F-NMDA (3 h), $p < 0.05$.

fibers of the retinular cells and the centrifugal fibers of the medulla in the optical lobe (Fig. 9B).

The immunohistochemical data indicated that myosins -Va and -VI, and synaptophysin were distributed in the antennal lobe (Fig. 10). The anti-myosin-VI staining recognized proteins from the pericellular and perinuclear regions of the interneurons (Fig. 10C and D). These regions were also stained blue with cresyl violet (Fig. 10A). The anti-myosin-Va staining revealed a similar pattern, and this myosin was also located in the glomerular fibers (Fig. 10E and F), which contain high zinc concentrations that may not allow for visualization by Neo-Timm histochemistry (Fig. 10B). However, synaptophysin localization was restricted to the interneurons (Fig. 10G and H).

As shown in Fig. 11A, the mushroom bodies are divided in the peduncle and calyx, which consists of the lip, collar, and basal rings, and in the non-compact and inner compact Kenyon cells. A myosin-Va antibody recognized proteins in the peduncle and calyx (Fig. 11C and D), which also contain high zinc concentrations (Fig. 11B), whereas synaptophysin localization was restricted to the Kenyon cells (Fig. 11E and F), visualized in blue by cresyl violet (Fig. 11A).

4. Discussion

An affinity-purified polyclonal antibody against chicken myosin-Va, an ancient myosin conserved from yeast to mammals

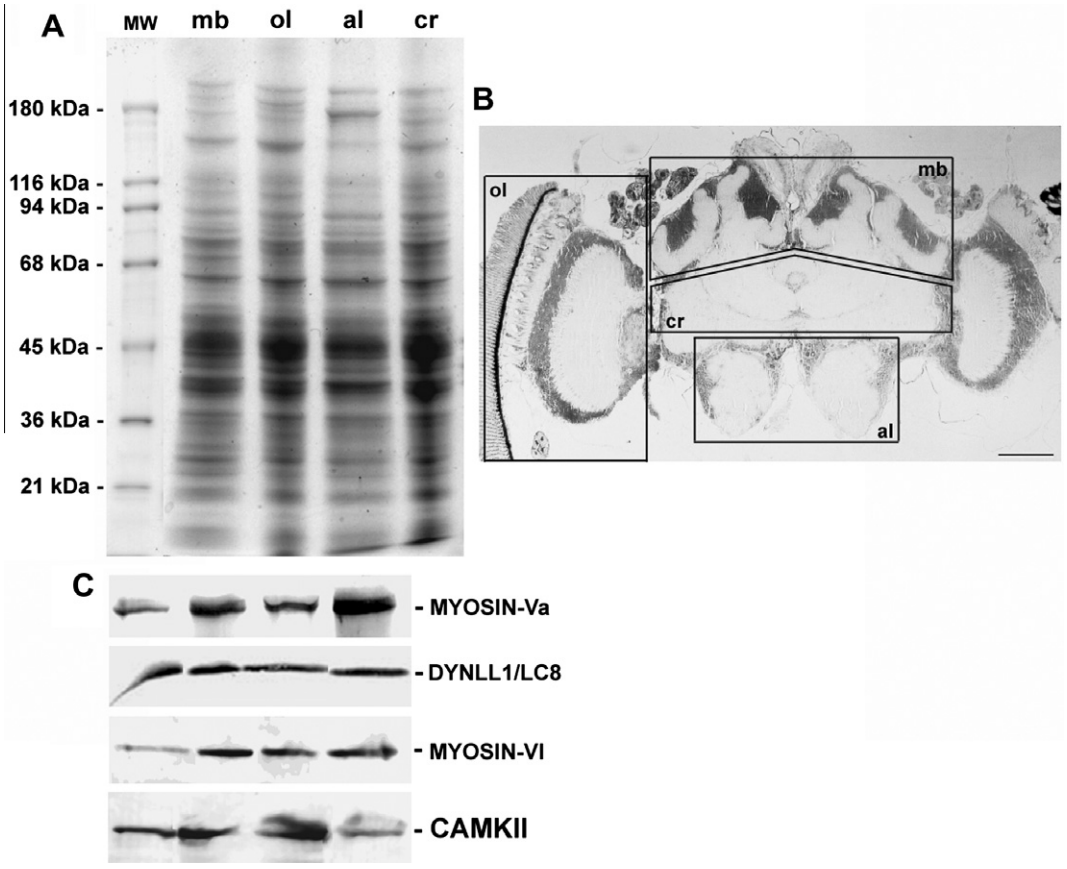

Fig. 7. Distribution of myosin-Va, myosin-VI, DYNLL1/LC8 and CaMKII in different regions of the honey bee brain. (A) A Coomassie blue-stained gel of the mushroom bodies (mb), optical lobes (ol), antennal lobes (al) and central region (cr), which are outlined in panel B by violet cresyl staining of a brain section (scale bar = 200 μm). The bottom panel shows Western blots of each brain region probed with antibodies against these proteins (C).

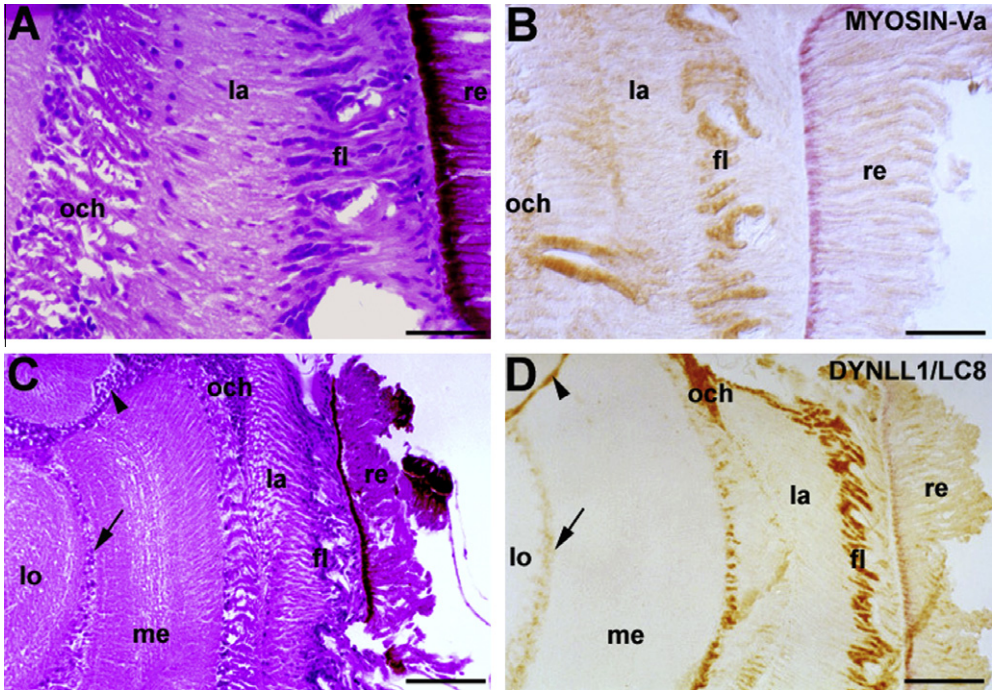

Fig. 8. Distribution of myosin-Va and DYNLL1/LC8 in the honey bee *Apis mellifera* optical lobe. (A and C) H&E staining shows the structures of the optical lobe, which are divided into retina (re), lamina (la), fenestrated layer (fl), outer chiasm (och), medulla (me) and lobula (lo). Immunolocalization of myosin-Va (B) and DYNLL1/LC8 (D). Both antibodies recognized proteins in the monopolar neurons of the fenestrated layer and cells of the outer chiasm. The anti-DYNLL1/LC8 antibody also stained cells of the inner chiasm (arrow) and outer compact Kenyon cells (arrowhead). Bars – A, B: 25 μm; C, D: 50 μm.

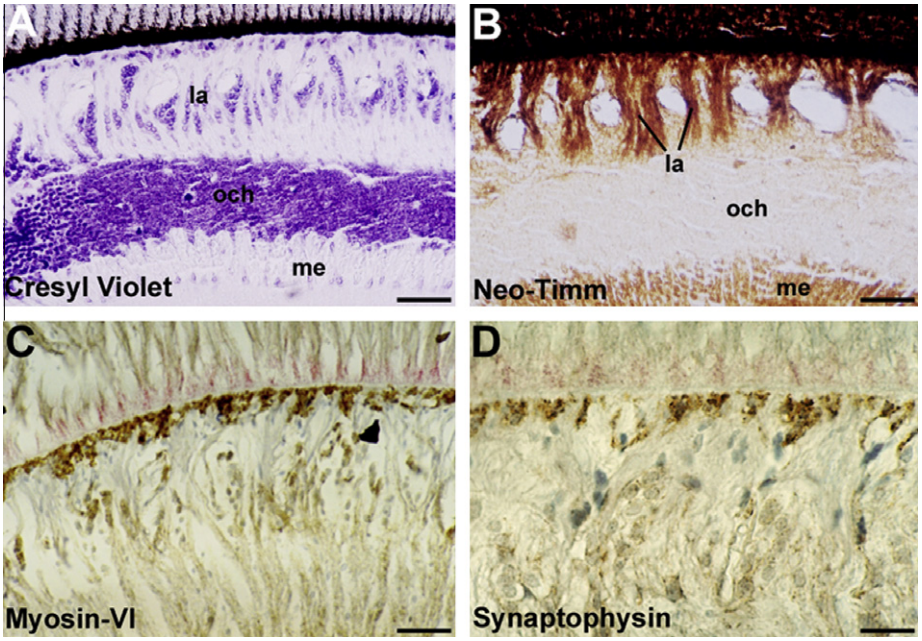

Fig. 9. Immunolocalization of myosin-VI and synaptophysin in the honey bee *Apis mellifera* optical lobe. (A) Cresyl violet staining and (B) Neo-Timm histochemistry show fibers of the lamina (la) and medulla (me) and cells of the outer chiasm (och) of the optical lobe. Immunolocalization of myosin-VI (C) and synaptophysin (D). Both antibodies recognized proteins in the monopolar neurons of the lamina and some cells of the outer chiasm. Bars – A, B: 30 μm; C, D: 12.5 μm.

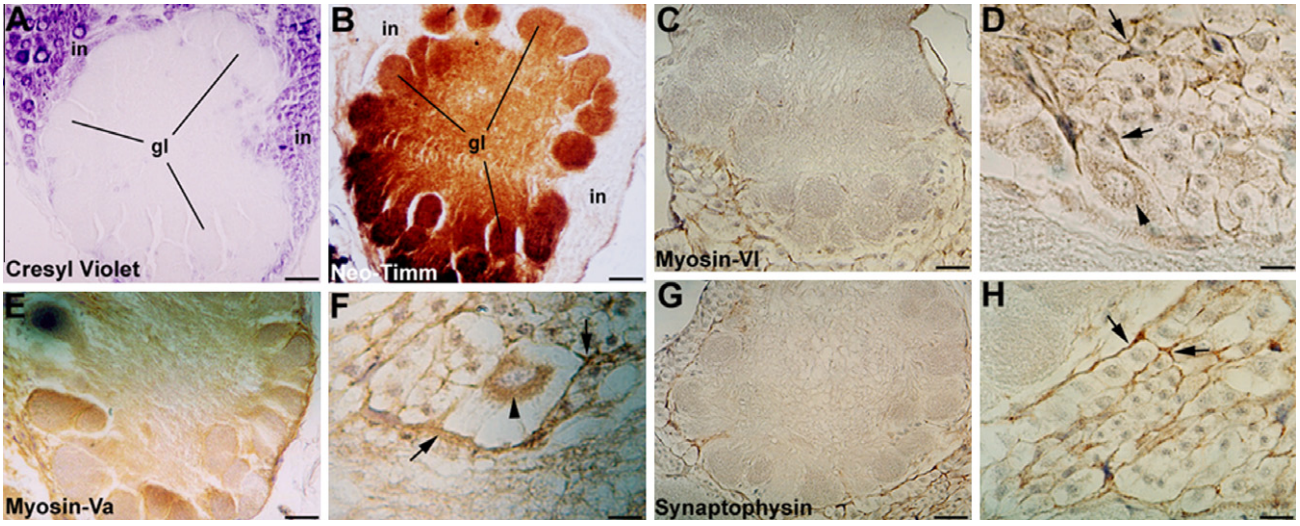

Fig. 10. Distribution of myosins -Va and -VI, and synaptophysin in the honey bee *Apis mellifera* antennal lobe. (A) Cresyl violet staining and (B) Neo-Timm histochemistry show the fibers of the glomerulus (gl) and interneurons (in) of the antennal lobe. Immunolocalization of myosin-VI (C and D), myosin-Va (E and F) and synaptophysin (G and H). The antibody against myosin-VI recognized proteins between the interneurons (arrows) and the cytoplasm in a perinuclear manner (arrowhead). This same distribution was found for myosin-Va, but the anti-myosin-Va staining also showed protein in the glomerulus fibers. Synaptophysin localization was restricted between the interneurons (arrows). Bars – A, B, C, E, G: 30 μm; D, F, H: 12.5 μm.

(Berg et al., 2001), was successfully used to identify its heavy chain in the honey bee brain and to immunolocalize this myosin in brain sections. Myosins -IIb, -VI and -IXb, cytoplasmic dynein intermediary chain (DIC74), light chain DYNLL1/LC8, CaMKII and SNARE proteins were also immunodetected in the honey bee brain. The DNA sequences of these immunodetected myosins and cytoplasmic dynein in the honey bee brain were found in the *A. mellifera* genome and in the genomes of other species (Odronitz et al., 2009).

Bioinformatic analyses using the Blastp tool showed a high level of sequence similarity for these proteins in the honey bee and vertebrates (*e*-value 0.0). In regards to myosin-Va, there is a UniGene record for an *A. mellifera* nucleotide sequence (Ame.1621, similar to myosin VA, heavy polypeptide 12, myoxin, LOC726456), the transcribed sequence of which matches the head domain of *D. melanogaster* myosin-V. Our results indicated myosin-Va was present in the honey bee nervous system in larvae and adult castes and subcastes using an antibody that also cross-reacts with myosin-V from the extruded axoplasm of the squid optical lobe (Tabb et al., 1998).

To examine the potential for cross-reactions between honey bee brain proteins and antibodies generated against vertebrate proteins, we probed Western blot of brain samples from rabbit, rat and honey bee with chicken brain myosin-Va and bovine brain CaMKII antibodies. The expression CaMKII gene has been previously reported in the honey bee brain by (Kamikouchi et al.,

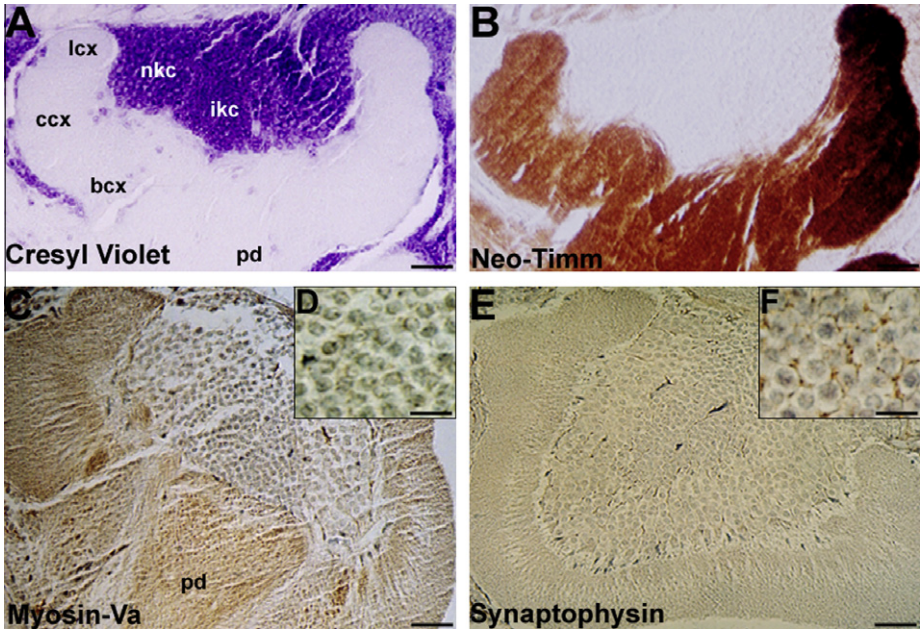

Fig. 11. Immunolocalization of myosin-Va and synaptophysin in the honey bee *Apis mellifera* mushroom bodies. (A) Cresyl violet staining and (B) Neo-Timm histochemistry show the structure of the mushroom bodies, which are divided into the peduncle (pd) and calyx, which consists of the lip (lcx), collar (ccx), and basal ring (bcx), and the Kenyon cells, which consists of non-compact (nkc) and inner compact cells (ikc). The antibody against myosin-Va (C) recognized protein in the peduncle and calyx, but not Kenyon cells (D). Synaptophysin localization (E) was restricted to the Kenyon cells (F). Bars – A, B: 30 μm; C, E: 30 μm; D, F: 12.5 μm.

2000). Moreover, microtubule- and actin-based motors, such as dynein and myosins (classes II, V, VI and IX), were immunodetected in the honey bee brain, which indicates that molecular motors and SNARE proteins could potentially be studied as neuronal targets in the honey bee nervous and visual systems. As recently reviewed by Hirokawa et al. (2010), the kinesin, dynein, and myosin superfamilies of molecular motors play fundamental roles in neuronal function. In addition to our findings that report dyneins and myosin-IIb and -IXb for the first time in the honey bee brain, other studies have shown that myosin-IXb is expressed in the rat brain (Chieregatti et al., 1998) and myosin-IIb is associated with synaptic function (Rex et al., 2010; Ryu et al., 2006).

In the worker honey bee brain, we have also shown the immunodetection of SNARE proteins involved in cellular trafficking of synaptic vesicles and neurotransmission, such as clathrin, syntaxin, SNAP25, munc18 and synaptophysin. Clathrin has been previously reported with myosins -V and -VI in synaptosomes prepared from honey bee brains and fractionated in a Percoll gradient (Silva et al., 2002), and myosin-Va has been immunolocalized by Calabria et al. (2010).

In this study, we obtained a honey bee brain P_2 fraction using the same protocol used to purify myosin-Va from chicken brains. In the vertebrate brain, a similar P_2 fraction showed that myosin-Va is associated with actin and fragments of the Golgi apparatus, mitochondria, endoplasmic reticulum and synaptic vesicle membrane (Evans et al., 1998). Our results showed that the P_2 fraction of the honey bee brain contains myosins -Va and -VI, DYNLL1/LC8, CaMKII, synaptotagmin and clathrin. These data provide new directions for future studies on the interactions between honey bee brain myosin-Va and other target proteins associated with its function. Vertebrate myosin-Va is found in synaptic vesicle preparations and forms stable complexes between synaptic vesicle proteins, such as synaptobrevin II, synaptophysin and syntaxin (Mani et al., 1994; Prekeris and Terrian, 1997; Watanabe et al., 2005).

While the direct mechanisms of melittin-induced myosin-Va overexpression have yet to be defined, a study has shown that this bee toxin binds to a myriad of calmodulin-binding proteins (Jarrett and Madhavan, 1991). Interestingly, melittin affects the calmodulin-dependent ATPase activity of chick brain myosin-Va (unpublished results). A more recent study demonstrates melittin attacks the plasma membrane of blood cells and induces death by loss of cytoplasmic contents. However, it remains to be determined whether this permeabilization allows release of higher molecular complexes like myosin-Va itself or whether a pro-survival response could induce protein overexpression. Similarly, the mechanisms underlying NMDA effects remain to be elucidated. A previous study showed myosin-Va levels increased in mammalian cell cultures treated with NMDA (Alavez et al., 2004). It is possible that this increase reflect a higher demand of vesicle and organelle trafficking to allow neuronal plasticity in response to NMDA. Finally, like kinesin, myosins -IIb and -Vb (Amparan et al., 2005; Hirokawa et al., 2010; Lei et al., 2001; Wang et al., 2008), it is also possible that myosin-Va be involved in trafficking of NMDA receptor subunits.

Mammals express the DYNLL1 and DYNLL2 isoforms that interact with myosin-Va and cytoplasmic dynein (Naisbitt et al., 2000; Pfister et al., 2006). DYNLL proteins are highly conserved throughout evolution, and more than 94% sequence identity exists between *D. melanogaster* and mammals (Patel-King and King, 2009; Wilson et al., 2001). The non-mammalian ortholog DYNLL1/LC8 was also immunodetected in the honey bee brain using an antibody raised against *Chlamydomonas* dynein light chain LC8 and an antibody raised against PIN. Both antibodies recognize DYNLL1/LC8 in honey bee, which reinforces that it is a conserved protein (Espindola et al., 2000; Jaffrey and Snyder, 1996; Odronitz et al., 2009). In the present study, after fractionation of the soluble honey bee brain fraction by gel filtration, Western blot indicated the presence of DYNLL1/LC8 throughout the eluted fractions, which suggested the co-elution of this protein with high molecular weight proteins, such as dynein and myosin-Va.

The biochemical and physicochemical properties of myosin-Va have been described, including the interaction of its head domain with actin, which is influenced by ATP and ADP (Nascimento

et al., 1996). The effect of ATP was also observed for myosin-Va from honey bee brain protein fractions. In fact, ATP induces the release of myosin-Va from F-actin, which allows it to remain in the supernatant, and the F-actin cytoskeleton is pelleted by centrifugation (Espindola et al., 1992; Tauhata et al., 2001). We also noted that the solubility of DYNLL1/LC8 increases similarly to myosin-Va in the presence of ATP. Future studies will determine if a physical interaction between these two proteins exist.

The distributions of CaMKII, DYNLL1/LC8, and myosins -Va and -VI in the honey bee brain indicated that these proteins are expressed in specific regions of the four dissected neuropils. In regard to CaMKII immunodetection, we found higher expression levels in the antennal lobe than in the other regions. The differentiation of the honey bee brain regions is reflected in the distribution of important kinases of the signal transduction system. Protein kinases A and C, CaMKII and inositol 1,4,5-trisphosphate receptor were expressed preferentially in the mushroom bodies (Kamikouchi et al., 2000, 1998; Muller, 1999).

It is possible that the distribution patterns of myosins, DYNLL1/LC8 and synaptophysin are associated with the functions of these proteins in these regions of the honey bee brain. Through immunolocalization analyses, myosin-Va was found in the optical and antennal lobes, and in the mushroom bodies. In the neuropils, myosin-Va was expressed in neurons and fibers in all of the honey bee brain regions evaluated. Myosin-Va studies in the vertebrate brain have also reported that it is localized in neurons and glial cells (Espindola et al., 1992; Martins et al., 1999; Tilelli et al., 2003).

In mushroom bodies, we also demonstrated that the localization of synaptophysin was restricted to the membrane space of Kenyon cells. This protein is an integral synaptic vesicle glycoprotein (Leube et al., 1987) and is widely used as a marker for synapses because it is distributed in presynaptic terminals (Li et al., 2010). In addition, myosin-Va was immunolocalized in the fibers of the mushroom bodies in a manner similar to the distribution of zinc in this honey bee brain region. The calyces receive projections from different areas of the brain, especially from the antennal and optical lobes (Cayre et al., 1998). Moreover, concerning spatial learning, the insect mushroom body is equivalent to the vertebrate hippocampus (Capaldi et al., 1999), where the zinc is more abundant in the brain (Slomianka, 1992; Zimmer, 1973). Our findings show for the first time that histochemically reactive zinc, as determined by the Neo-Timm method, is present in specific regions of the honey bee brain.

The optical lobe is involved in the visual and sensorial activities, while the mushroom bodies constitute the main memory center where complex local synaptic circuits have been previously described (Kamikouchi et al., 1998). Therefore, the myosin-Va localization data indicate that it is widely distributed in the brain. This finding agrees with previous reports, which have used myosin-Va as a neuronal marker for immunohistochemical studies of the honey bee brain (Calabria et al., 2010) and to map brain structures in vertebrates (Martins et al., 1999; Tilelli et al., 2003).

In general, DYNLL1/LC8 and myosin-Va showed similar patterns of immunolocalization. Differences in the staining patterns were found in the monopolar neurons of the fenestrated layer and in the outer and inner chiasms of the optical lobe, whereas myosin-VI and synaptophysin were localized to the retina and monopolar neuron of the lamina. Moreover, zinc was amply distributed on the long fibers of the lamina and fenestrated layer, which were also enriched in DYNLL1/LC8 and myosin-Va. The cells of the optical lobe subregions have been shown to be immunoreactive to the serotonin, GABA and catecholamine neurotransmitters (Meyer et al., 1986; Nassel et al., 1986).

Although our data for the antennal lobe indicated that myosin-VI and synaptophysin were restricted to the interneurons, myosin-Va was only found in the fiber terminal fields of the glomeruli, as also revealed for the zinc immunostaining. These findings can be explained by the composition and function of this neuropil, which transmits information to the mushroom bodies and other lobes (Galizia and Menzel, 2000; Kloppenburg, 1995; Menzel and Muller, 1996; Nassel et al., 1986).

4.1. Conclusion

The results obtained in our study indicated that myosin-Va is present in the honey bee nervous system in the larvae and adult castes and subcastes. We also showed that DYNLL1/LC8, and myosins -IIb, -VI and -IXb are present in the adult brain, as well as SNARE proteins, such as CaMKII, clathrin, syntaxin, SNAP25, munc-18, synaptophysin and synaptotagmin. Our study revealed increased expression levels of myosin-Va classically associated with neuron function and plasticity when we challenged honey bee brains with melittin, a naturally occurring bee toxin, and NMDA, a synthetic excytotoxin, and open perspective of new studies to determine the mechanisms underlying myosin-Va overexpression and if this is a pro-survival response. Based on the immunolocalization and histochemistry data, the optical and antennal lobes and the mushroom bodies exhibited distinct and punctate distributions of myosins, DYNLL1/LC8 and synaptophysin. Therefore, this study indicates that the identification and subcellular localization of these molecular motors and SNARE proteins in the honey bee brain should be investigated further because myosins and dynein are potentially involved in vesicle transport during synaptic processes of specific areas of the honey bee brain.

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